

Identification of *in vitro* upregulated genes in a modified live vaccine strain of *Edwardsiella ictaluri* compared to a virulent parent strain

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ABSTRACT

Using PCR-select subtractive cDNA hybridization technique, 41 *in vitro* expressed sequence tags (ESTs) were isolated from a modified live vaccine strain (AQUAVAC-ESC[®], formerly RE-33) vs a virulent parent strain (EILO) of *Edwardsiella ictaluri*. Transcriptional levels of the 41 *in vitro* ESTs in the vaccine strain and the virulent strain were then evaluated by quantitative PCR (qPCR). The qPCR results revealed that 33 ESTs were consistently upregulated at least 3-fold in the modified live vaccine strain compared to the virulent parent strain. Of the 33 upregulated ESTs, 11 were upregulated greater than 5-fold. The 41 ESTs were found to be homologues of genes involved in protective immunity (22%), adhesion (7%), cell growth and survival (20%), signaling (7%), metabolism (5%), and transcriptional regulation (5%). However, putative functions of 20% of the genes identified are currently unknown. Putative roles of some of the *in vitro* upregulated genes in protective immunity induced by the vaccine strain are discussed.

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1. Introduction

Enteric septicemia of catfish (ESC), the most prevalent disease affecting farm-raised channel catfish, *Ictalurus punctatus*, is caused by *Edwardsiella ictaluri*, a facultative intracellular Gram-negative flagellated bacterium akin to phylogenetically related *Salmonella* [1,2]. ESC is generally an acute septicemia that develops very quickly, especially in the temperature range of 22–28 °C. Signs of the disease have been observed within 2 days after immersion challenge and heavy mortalities have been reported as early as 4 days after infection [1,3,4].

Initial efforts to control ESC were based on feeding the infected fish with antibiotic medicated food [5]. However, this practice is expensive and usually ineffective as sick fish do not eat. Furthermore, fish have developed resistance to approved food fish antibiotics such as oxytetracycline, florfenicol, and ormetoprim-sulphamethoxine

[6,7]. Therefore, alternative strategies are urgently needed to control ESC. A modified live *E. ictaluri* vaccine using a rifampicin-resistant strategy has been successfully developed by Klesius and Shoemaker [8]. This strategy relies on the ability of rifampicin to induce the appearance of rough mutants. It has been demonstrated that the rifampicin-resistant strain of *E. ictaluri* was unable to cause ESC, but was able to stimulate protective immunity in catfish [8]. The *E. ictaluri* RE-33 was patented (US Patent No. 6,019,981) and licensed to Intervet/Schering-Plough Animal Health by the US Department of Agriculture, Agricultural Research Service. This modified live ESC vaccine was marketed by Intervet/Schering-Plough Animal Health as a licensed vaccine (AQUAVAC-ESC[®]) through immersion of 7–10-day post-hatch channel catfish.

Live vaccines have played a critical role since the beginning of vaccinology. In fact, the very first vaccination experiment in the Western world was inoculation of a boy with the milder cowpox virus to protect against the deadly smallpox [9]. Live vaccines are very effective due to the fact that live vaccines mimic the route of entry of many pathogens and stimulate protective immune responses in

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the host. The major concerns associated with live vaccine are due to two safety issues: (1) the live vaccine might revert back to a virulent organism; (2) the live vaccine might cause disease in immune compromised individuals and other non-target organisms sharing the aquatic environment. Other technologies for making vaccines besides live attenuated vaccines include whole killed microorganisms and recombinant subunit vaccines [10,11].

Recombinant DNA techniques have enormous potential for the development of inexpensive, safe and efficacious vaccines for the aquaculture industry. However, the low-cost systems for recombinant protein expression (especially *Escherichia coli*, but also yeast) may generate misfolded or incorrectly processed membrane antigens that fail to protect, while more complex insect and mammalian tissue culture cells are prohibitively expensive from a production standpoint [11]. Furthermore, recombinant subunit antigens may generate less than optimal cytotoxic T-cell responses, which are important for the clearance of intracellular pathogens.

To develop effective novel vaccines, the most important step is to understand the host–pathogen interaction as well as the molecular differences between modified live vaccine strain and virulent strain. Such knowledge would open almost unlimited possibilities to develop new immunization strategies, such as recombinant microorganisms, recombinant polypeptides, bacterial or viral vectors, synthetic peptides, natural or synthetic polysaccharides, and plasmid DNA. The increasing use of AQUAVAC-ESC[®] throughout the catfish industry warrants a comparative gene regulation study between the modified live vaccine strain and the virulent parent strain to understand the differences between the two strains at transcriptional level.

Different methods have been successfully used by researchers to generate differentially expressed genes between two samples, such as cDNA library screening, differential display, and cDNA microarray. However, since its first introduction [12], suppression subtractive hybridization (SSH) has been widely used by researchers in different fields of science [13–15] due to two major reasons: (1) SSH is more cost effective compared to microarray and (2) SSH does not require previously known genome information for the organism. SSH has been proven to be a powerful method to isolate differentially transcribed genes between bacteria strains [15] and fungi strains [16]. In this study, 41 differentially expressed sequencing tags (ESTs) were obtained from the modified live vaccine vs virulent parent library. The putative functions of the differentially transcribed genes are discussed.

2. Materials and methods

2.1. Bacterial isolates

The virulent parent *E. ictaluri* strain, EILO, was originally isolated from walking catfish, *Clarias batrachus* L. from Thailand [17]. Previously attempt to use virulent *E. ictaluri* strain isolated from channel catfish to develop modified

live vaccine strain through rifampicin-resistant strategy failed (unpublished data). The rifampicin-resistant mutant, RE-33, was derived from the virulent parent strain through 33 passages on brain heart infusion agar (BHI) (Difco Laboratories, Detroit, MI, USA) supplemented with increasing concentrations of rifampicin [3-(4-methylpiperazinyl-im-imomethyl) rifamycin SV] (Sigma Chemical Company, St. Louis, MO, USA) to a final concentration of 320 µg/ml. Since 1999, RE-33 has been used successfully as a modified live vaccine under the label of AQUAVAC-ESC[®] (Intervet/Schering-Plough Animal Health, Elkhorn, NE, USA) for channel catfish. Stock cultures were stored at –80 °C in BHI with 10% glycerol. The vaccine strain was corroborated by passing the cells once from the stock to BHI containing rifampicin (320 µg/ml). Cells were grown on BHI at 26 °C for 48 h prior to RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated from modified live vaccine and parent bacterial cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Total RNA was DNase treated with DNA-free (Ambion, Austin, TX), and the absence of contaminating DNA was verified by PCR using total RNA as template and gene-specific primers of *E. ictaluri* phosphoserine transaminase (GenBank accession no. AF110153). The forward primer used in the PCR was Eic-F (5'-ACTTATCGCCCTCGCAACTC-3') and the reverse primer was Eic-R (5'-CCTCTGATAAGTGGT-TCTCG-3'). All total RNAs were quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). Total RNAs were resuspended in distilled water and cDNA synthesis was immediately performed. The first strand cDNAs used for quantitative PCR were synthesized using AMV reverse transcriptase (Invitrogen, Carlsbad, CA). For subtractive library construction, total RNAs were pooled from the three individual total RNA preparations from RE-33 or EILO cells that were grown on different plates. cDNAs were then synthesized from the pooled total RNAs using PCR-selected cDNA Subtraction Kit (Clontech, Palo Alto, CA) as described by the manufacturer. The cDNAs that contain specific transcripts are referred to as "testers" (i.e. from modified live vaccine strain) and the reference cDNAs are referred to as "drivers" (i.e. from virulent parent strain). The double-stranded cDNAs of both testers and drivers were digested with *RsaI* to create smaller blunt-ended fragments to be used as testers or drivers according to the manufacturer's instruction (Clontech, Palo Alto, CA). The tester cDNAs were then subdivided into two portions (A and B) and modified by ligating with cDNA adaptors 1 and 2 (provided by the Kit), respectively.

2.3. Construction of subtractive cDNA library

Two-step subtractive hybridizations were performed according to procedures used previously [18]. Briefly, in the first step hybridization, two primary hybridization reactions (A and B) were formed by adding excess amounts of unmodified driver cDNA to separate portions A and B of tester cDNA samples at a 50:1 ratio. The samples were

denatured for 2 min at 98 °C and allowed to anneal for 8 h at 68 °C. The remaining single-stranded, adaptor-ligated tester cDNAs were dramatically enriched in each hybridization reaction for overexpressed sequences because non-target cDNAs present in the tester and driver formed hybrids. For the second step hybridization, A and B primary hybridization reaction solutions were mixed together without denaturing. These new hybrids were double-stranded tester molecules with different 5'-ends corresponding to the sequences of two different adaptors. Freshly denatured driver DNA was added to the reaction without denaturing the subtraction mix to further enrich new double-stranded tester molecules that are differentially expressed. After filling in the adapter ends with DNA polymerase, overexpressed sequences (tester cDNA) had different annealing sites on their 3'- and 5'-ends. The molecules were then subjected to suppression subtraction PCR as described by the manufacturer (Clontech, Palo Alto, CA). The PCR products were then cloned into pGEM-T easy vector (Promega, Madison, WI). Plasmids were then transformed into One Shot[®] TOP10 competent cells (Invitrogen, Carlsbad, CA). Transformed cells were then plated out on Luria–Bertani (LB) plates containing ampicillin (100 µg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (40 µg/ml).

2.4. Colony culture and sequencing

From the library, a total of 96 white colonies were picked to grow overnight in LB broth in the presence of ampicillin (100 µg/ml) and glycerol (5%) in the Innova[™] 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ) at 37 °C and 235 rpm settings, respectively. Colony culture was then frozen and sent to USDA-ARS Mid South Genomic Laboratories in Stoneville, MS for sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were then analyzed using the National Center for Biotechnology Information (NCBI) BLAST program to search for homologies based on deduced protein sequences.

2.5. Primer design and quantitative PCR

Sequencing results of different clones were used to design gene-specific primers by using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For each cDNA sample, *E. ictaluri* 16S ribosomal RNA (GenBank accession no. EU541494) primers were included as an internal control to normalize the variation of cDNA amount. Primers used for the amplification of the 16S rRNA gene was 16S-F (5'-CAGCCACACTGGAAGTGA-3') and 16S-R (5'-TTAGCCGGTGCTTCTTGT-3'). All qPCRs were performed on an Applied Biosystems 7000 Real-Time PCR System (ABI, Foster City, CA) using Platinum[®] SYBR[®] Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a total volume of 12.5 µl. The qPCR mixture consisted of 1 µl of cDNA, 0.5 µl of 5 µM gene-specific forward primer, 0.5 µl of 5 µM gene-specific reverse primer and 10.5 µl of 1 × SYBR Green SuperMix. The qPCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycle of 95 °C for 15 s and 60 °C for 1 min. All qPCRs were run in duplicate

for each cDNA sample and three *E. ictaluri* cDNA samples were analyzed by qPCR.

2.6. qPCR data analysis

The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (C_t) of the sample by that of the 16S rRNA, the calibrator or internal control, as per the formula: $\Delta C_t = C_t(\text{sample}) - C_t(\text{calibrator})$. The relative transcriptional level of a specific gene in the modified live vaccine strain compared to that in the virulent parent strain was then calculated by the formula $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_t(\text{RE-33}) - \Delta C_t(\text{EILO})$ as described previously [19]. Data were analyzed by analysis of variance (ANOVA) using SigmaStat statistical analysis software (Systat Software, San Jose, CA).

3. Results

3.1. Characteristics of the subtractive cDNA library

A total of 96 clones were obtained from the subtractive library. Of the 96 clones, 67 contained inserts. Sequencing results revealed that these 67 ESTs represented 41 different genes (Table 1). All ESTs listed in Table 1 have been deposited in the GenBank dbEST under accession numbers GR487953–GR487993. Of the 41 ESTs, 40 shared homologies with bacterial proteins. Of the 40 bacterial ESTs, seven shared homology with *E. ictaluri* or *E. tarda* proteins, eight shared homology with *E. coli* or *E. fergusonii* proteins, five shared homology with *Serratia proteamaculans* proteins, three shared homology with *Yersinia* sp. proteins, three shared homology with *Pectobacterium carotovorum* proteins, two shared homology with *Salmonella enterica* or *S. typhimurium* proteins, three shared homology with *Yersinia* proteins, two shared homology with *Klebsiella pneumoniae* proteins, and two shared homology with *Citrobacter koseri* proteins (Table 1). The biggest insert size was 968 bp (GR487989) and the smallest was 110 bp (GR487978). The average insert size of the 41 ESTs was 290 bp (Table 1).

3.2. Transcriptional profiling of the 41 ESTs in the modified live vaccine strain

To determine whether the transcriptional levels of the 41 ESTs isolated from the subtractive library were upregulated in the modified live vaccine strain, gene-specific primers for the 41 ESTs were designed (Table 2) for qPCR. The qPCR results revealed that 33 ESTs were transcribed at least 3-fold higher in the modified live vaccine strain compared to that in the virulent parent strain (Fig. 1). One EST, GR487971 (TnpA-like protein), was consistently upregulated greater than 10-fold. Of the 33 consistently upregulated ESTs, 11 were upregulated greater than 5-fold in the modified live vaccine strain, including EST GR487953 (adenylosuccinate synthetase purA), EST GR487955 (sensory histidine kinase AtoS), EST GR487956 (putative cytoplasmic protein), EST GR487957 (DNA-binding transcriptional activator CadC), EST GR487958 (penicillin-binding protein 2), EST GR487965

(putative cytoplasmic protein YgbA), EST GR487966 (NADH:ubiquinone oxidoreductase subunit C/D), EST GR487981 (vitamin B12 outer membrane transporter), EST GR487982 (hypothetical protein KPN_02837), EST GR487988 (type IV B pilus protein), and EST GR487975 (phosphatidylserine synthase) (Fig. 1). The transcriptional levels of 22 ESTs in the vaccine strain were at least 3-fold greater than that in the virulent strain. However, the transcriptional levels of seven ESTs in the vaccine strain were not consistently upregulated, including EST GR487973 (hypothetical protein), EST GR487974 (endonuclease/exonuclease/phosphatase family protein), EST GR487989 (EsrA), EST GR487990 (Ni,Fe-hydrogenase I large subunit), EST GR487991 (putative membrane protein), EST GR487992 (intracellular septation protein A), and EST GR487993 (putative threonine dehydratase) (Fig. 1).

3.3. Classification of the 41 genes isolated from the subtractive library

The 41 genes isolated from the subtractive library were classified in terms of their putative functions. One-third of the genes identified were either involved in protective immunity or adhesion, including adenylosuccinate synthetase (*purA*), glucose-6-phosphate isomerase, penicillin-binding protein 2, D-alanyl-D-alanine carboxypeptidase, *E. coli* *dacA* gene, penicillin-binding protein 5, phosphatidylserine synthase, flagellar hook-associated protein, heat shock protein 90, 33 kDa chaperonin, outer membrane protein assembly factor YaeT, type IV B pilus protein, *EsrA*, and glycosyltransferase (Table 3). 20% of the genes identified were important for cell growth and survival, including 6-phosphofructokinase, aerobic respiration control sensor protein ArcB, vitamin B12 outer membrane transporter, MscS mechanosensitive ion channel, putative permease, 50S ribosomal protein L14, and NADH:ubiquinone oxidoreductase subunit C/D (Fig. 2). The 41 ESTs identified also included genes involved in cell signaling (7%), metabolism (5%), and transcriptional regulation (5%). However, 20% of the genes identified were putative or hypothetical proteins (Fig. 2).

4. Discussion

Enteric septicemia of catfish is the most prevalent disease affecting farm-raised channel catfish [20] and live attenuated *E. ictaluri* vaccines have been shown to be efficacious in protecting catfish through immersion immunization [21–24]. However, the molecular mechanisms involved in the protective immunity induced by this modified vaccine strain was unknown. Therefore, the purpose of this study was to understand the *in vitro* difference between the two strains at transcriptional level. Using PCR-select subtractive cDNA hybridization and qPCR techniques, 33 ESTs were found to be consistently upregulated at least 3-fold in the modified live vaccine strain compared to the virulent parent strain. The following is a brief discussion on some of the upregulated genes identified in the modified live vaccine strain.

EST GR487953 shared 100% homology with adenylosuccinate synthetase (*purA*) of *E. ictaluri* (*e* value = $5e-32$). Adenylosuccinate synthetase *purA* was found to be consistently upregulated greater than 5-fold in the modified live vaccine strain compared to that in the virulent parent strain. The function of adenylosuccinate synthase *purA* gene is to convert inosine monophosphate (IMP) to adenosine monophosphate (AMP) and its inactivation produces a requirement for adenine [25]. Mutation of the *purA* gene has previously been shown to attenuate *Salmonella enterica* serovar *typhimurium* [26], *S. enterica* serovar *typhi* [27], and *E. ictaluri* [28]. However, it has also been reported that *purA* mutants of *S. enterica* are not good vaccine candidates providing no protective immunity [26,29,30]. Our results of *purA*'s upregulation (5–8-fold) in the vaccine strain supports the notion that *purA*'s overexpression, not deletion, might provide better protective immunity.

EST GR487958 shared 82% homology with penicillin-binding protein 2 (PBP2) of *Sodalis glossinidius* (Enterobacteriaceae) (*e* value = $5e-13$). Penicillin-binding proteins (PBPs) are conserved proteins that play a major role in peptidoglycan biosynthesis [31]. Their possible role as an immunogenic protein has been recently analyzed in *Neisseria meningitidis*, the causing agent of meningococcal disease. Vaccination with purified recombinant penicillin-binding protein and purified anti-penicillin-binding protein rabbit IgG antibody is able to confer protection against experimental meningococcaemia in mice [32]. Our results revealed that the transcriptional level of PBP2 in the modified live vaccine strain was consistently transcribed 5-fold greater than that in the virulent strain, suggesting that PBP2 might also play an important role in the protective immunity induced by the vaccine strain.

EST GR487976 shared 67% homology with flagellar hook-associated protein flgK of *Yersinia mollaretii* (Enterobacteriaceae) (*e* value = $2e-22$). Flagellar hook-associated proteins are essential for flagellar filament formation [33]. It has been demonstrated that FlagK of *Campylobacter jejuni*, a commensal Gram-negative motile bacterium commonly found in chickens and a frequent cause of human gastrointestinal infections, is required for assembly of the flagellar secretory apparatus [34]. Deletion mutant of flgK expressing only the hook showed diminished motility of *C. jejuni* [34]. Flagella primarily serve as organelles for locomotion in bacteria (swimming in liquid and swarming motility on solid media). In addition to locomotion, flagella are also potent immunostimulatory molecules that induce proinflammatory cytokine production [35]. Our result of consistent upregulation (3–6-fold) of flgK homologue in the modified live vaccine strain suggests that overexpression of flgK might also play an important role in the protective immunity induced by the vaccine strain.

EST GR487954 shared 96% homology with glucose-6-phosphate isomerase (G6PI) of *E. tarda* (*e* value = $8e-33$). G6PI, also known as phosphohexose isomerase, catalyzes the interconversion of fructose-6-phosphate and glucose-6-phosphate. G6PI is identical with neuroleukin, autocrine motility factor, and maturation factor [36]. It has been reported to be secreted by lectin-stimulated T-cells and to induce Ig synthesis and stimulate cell migration and the

Table 1

List of genes isolated from the *E. ictaluri* modified live vaccine strain RE-33 vs virulent strain AL93-58 subtractive cDNA library.

| Clone no. | Accession no. | Protein homology | Protein accession no. | Organism | Identity (%) | Score bits | e value | Insert (bp) |
|-----------|---------------|--|-----------------------|--|--------------|------------|---------|-------------|
| 1 | GR487953 | Adenylosuccinate synthetase (purA) | AAB86714 | <i>Edwardsiella ictaluri</i> | 100 | 140 | 5e–32 | 200 |
| 2 | GR487954 | Glucose-6-phosphate isomerase | BAE19909 | <i>Edwardsiella tarda</i> | 96 | 142 | 8e–33 | 199 |
| 6 | GR487955 | Sensory histidine kinase AtoS | YP_00145214_2 | <i>Citrobacter koseri</i> | 59 | 55.5 | 9e–25 | 469 |
| 7 | GR487956 | Putative cytoplasmic protein | NP_460658 | <i>Salmonella typhimurium</i> | 65 | 6.8 | 33.5 | 141 |
| 10 | GR487957 | DNA-binding transcriptional activator CadC | YP_001479993 | <i>Serratia proteamaculans</i> | 83 | 75.1 | 2e–12 | 133 |
| 11 | GR487958 | Penicillin-binding protein 2 | YP_454477 | <i>Sodalis glossinidius</i> | 82 | 77 | 5e–13 | 211 |
| 12 | GR487959 | Aerobic respiration control sensor protein ArcB | ZP_03830685 | <i>Pectobacterium carotovorum</i> | 73 | 119 | 1e–25 | 251 |
| 13 | GR487960 | D-Alanyl-D-alanine carboxypeptidase | ZP_02221171 | <i>Yersinia pestis biovar Orientalis</i> | 88 | 89.4 | 1e–16 | 351 |
| 15 | GR487961 | IS1N transposase | YP_001965465 | <i>Escherichia coli</i> | 60 | 67.4 | 4e–23 | 325 |
| 16 | GR487962 | Hypothetical protein PJE062_5242 | YP_002680152 | <i>Pseudovibrio sp.</i> | 27 | 66.2 | 5e–09 | 233 |
| 17 | GR487963 | 6-Phosphofructokinase | ZP_03833363 | <i>Pectobacterium carotovorum</i> | 88 | 59.3 | 1e–07 | 208 |
| 20 | GR487964 | IS630 transposase | YP_001745145 | <i>Escherichia coli</i> | 90 | 85.1 | 2e–15 | 124 |
| 22 | GR487965 | Putative cytoplasmic protein YgbA | ACL13586 | <i>Salmonella enterica</i> | 78 | 99.0 | 1e–19 | 197 |
| 23 | GR487966 | NADH:ubiquinone oxidoreductase subunitC/D | ZP_03830189 | <i>Pectobacterium carotovorum</i> | 100 | 79.0 | 1e–13 | 157 |
| 24 | GR487967 | Glycosyltransferase | YP_432363 | <i>Hahella chejuensis</i> | 47 | 56.2 | 1e–06 | 272 |
| 25 | GR487968 | Hypothetical protein UTI89_C3752 | YP_542720 | <i>Escherichia coli</i> | 94 | 64.7 | 3e–09 | 116 |
| 27 | GR487969 | <i>E. coli</i> dacA gene, penicillin-binding protein 5 | X06479 | <i>E. coli</i> | 69 | 116 | 7e–23 | 373 |
| 32 | GR487970 | DeoR family transcriptional regulator | YP_001480073 | <i>Serratia proteamaculans</i> | 80 | 116 | 6e–25 | 220 |
| 33 | GR487971 | TnpA-like protein | AAP70294 | <i>Escherichia coli</i> | 81 | 218 | 3e–55 | 660 |
| 34 | GR487972 | Predicted integrase | YP_002330279 | <i>Escherichia coli</i> | 58 | 234 | 3e–71 | 709 |
| 37 | GR487973 | Hypothetical protein | ZP_03317970 | <i>Providencia alcalifaciens</i> | 28 | 52 | 2e–05 | 529 |
| 38 | GR487974 | Endonuclease/exonuclease/phosphatase family | ZP_03028836 | <i>Escherichia coli</i> | 82 | 209 | 7e–53 | 373 |
| 44 | GR487975 | Phosphatidylserine synthase | AAT77727 | <i>Edwardsiella ictaluri</i> | 98 | 150 | 3e–35 | 207 |
| 45 | GR487976 | COG1256: Flagellar hook-associated protein | ZP_00824951 | <i>Yersinia mollaretii</i> | 67 | 108 | 2e–22 | 232 |
| 46 | GR487977 | Heat shock protein 90 | YP_001477370 | <i>Serratia proteamaculans</i> | 78 | 110 | 4e–23 | 216 |
| 47 | GR487978 | Outer membrane protein assembly factor YaeT | YP_002381423 | <i>Escherichia fergusonii</i> | 91 | 75.1 | 2e–12 | 110 |
| 49 | GR487979 | MscS mechanosensitive ion channel | YP_001175797 | <i>Enterobacter sp.</i> | 83 | 86.7 | 7e–16 | 148 |
| 52 | GR487980 | Zinc finger protein 184 (Krueppel-like) | XP_001601912 | <i>Nasonia vitripennis</i> | 34 | 33.9 | 5.3 | 330 |
| 53 | GR487981 | Vitamin B12 outer membrane transporter (btuB) | YP_001454553 | <i>Citrobacter koseri</i> | 68 | 76.3 | 9e–13 | 156 |
| 54 | GR487982 | Hypothetical protein KPN_02837 | YP_001336477 | <i>Klebsiella pneumoniae</i> | 62 | 47 | 6e–04 | 161 |
| 58 | GR487983 | IS1400 transposase A | YP_001004058 | <i>Yersinia enterocolitica</i> | 64 | 96.3 | 9e–19 | 437 |
| 63 | GR487984 | Putative permease | YP_002240777 | <i>Klebsiella pneumoniae</i> | 90 | 35.0 | 2.4 | 290 |
| 64 | GR487985 | 50S ribosomal protein L14 | NP_671292 | <i>Yersinia pestis</i> | 100 | 134 | 2e–30 | 366 |
| 65 | GR487986 | Hypothetical protein | YP_002935192 | <i>Edwardsiella ictaluri</i> | 100 | 64.7 | 3e–09 | 177 |
| 66 | GR487987 | 33 kDa chaperonin | ZP_01682893 | <i>Vibrio cholerae</i> | 80 | 63.5 | 6e–09 | 290 |
| 68 | GR487988 | Type IV B pilus protein | YP_001476558 | <i>Serratia proteamaculans</i> | 90 | 82.8 | 1e–14 | 137 |
| 69 | GR487989 | EsrA | ABC60089 | <i>Edwardsiella ictaluri</i> | 89 | 580 | 6e–164 | 968 |
| 71 | GR487990 | Ni,Fe-hydrogenase I large subunit | ZP_00134404 | <i>Actinobacillus pleuropneumoniae</i> | 80 | 146 | 7e–34 | 260 |
| 80 | GR487991 | Putative membrane protein | AAT47206 | <i>Edwardsiella ictaluri</i> | 100 | 76.6 | 7e–13 | 331 |
| 81 | GR487992 | Intracellular septation protein A | YP_001478906 | <i>Serratia proteamaculans</i> | 82 | 119 | 9e–26 | 364 |
| 85 | GR487993 | Putative threonine dehydratase | AAY43642 | <i>Edwardsiella tarda</i> | 73 | 82.4 | 2e–19 | 255 |

Table 2

Gene-specific primers used in qPCR.

| Clone no. | Accession no. | Forward primer (5'–3') | Reverse primer (5'–3') |
|-----------|---------------|--------------------------|-------------------------|
| 1 | GR487953 | TGAAGTACTACACGTCGATGC | GGCACCTTCGAACATCACAT |
| 2 | GR487954 | TGGTAATCCACGACCTGACC | GCTGATCGGCATCTGGTATAA |
| 6 | GR487955 | CCGGGAAGCTGACCTCTT | GCAATCTCACCACCATCAAC |
| 7 | GR487956 | CAGGAGCAAAATGGCAAATC | ATACAGGCTCTCCGATGAA |
| 10 | GR487957 | TGTCAATCAGCGCCACTATC | CTGAGCAGGTTATCCGGTGT |
| 11 | GR487958 | ACCAACTTGATGCGGTTTTC | GATCGTAACCCCTTTCGTGA |
| 12 | GR487959 | CGGTGCTAGAGGATGGAGAG | TCCAGCACCGATAGATAGCC |
| 13 | GR487960 | GTGGTGCTGAACGAAGTGAA | CGTAGCGGGAGCCTTACTTA |
| 15 | GR487961 | CCCCTAAGCTCGGAGTTAAA | CATGAGCAACAGGGATGATG |
| 16 | GR487962 | GCAGTTGTCAGATGGGTCTC | GGGTTTGGAATGAAGATTGC |
| 17 | GR487963 | CATCGATGCGGTTGAAAATA | TGAAGCCAACCTTTCCCTTG |
| 20 | GR487964 | GAGTGACGATGCGCTTTTG | CACAGAATACCTGTGTTTTACGA |
| 22 | GR487965 | CAGAATAGGGTGATGCCACA | GCATGCAAACAGTGTCCAAT |
| 23 | GR487966 | CTGTCTGGTGTCGCACTTGA | CAAAAACCGAATCCTTATCCA |
| 24 | GR487967 | TGAACATAGCCACGTTGAA | ATAGCAAGCGTTGGCAGATT |
| 25 | GR487968 | CCAAAGAGGCTAAGAAGACTAAGG | GTTGCAACCCAGCTCTTTGAG |
| 27 | GR487969 | GTTTCATCGCTGGTTTGTT | GCACCACTTCAACAACCTGA |
| 32 | GR487970 | TTTCGATCGGCTATTCTGG | TTTCCGGCCAAATTTACTG |
| 33 | GR487971 | ACTCTGCCGATCTTGAGGAA | GCTGGAACCATGGACTGGT |
| 34 | GR487972 | TCCAGCCTTACGCGAGTATT | GCAATAGAGGCTGGCAGAAC |
| 37 | GR487973 | GACAGCATCATCGTTTGTTG | ACTGACGATCACTGTGCTG |
| 38 | GR487974 | TCTGCTCACCAGATAGGGTCT | CGGCTCATCCGATCTATTGT |
| 44 | GR487975 | GCGGATACACTCCAGTTCGT | GAAAGGCATGTGGGTAGACG |
| 45 | GR487976 | TTATCGGTAATGGCGTCACA | GGTACTGTCCGCCATCAAGT |
| 46 | GR487977 | ATATCGCTCTGCTGTGCGAG | AAGTCATGGCGATGTGTTT |
| 47 | GR487978 | CCCCACCTGAAAGCTGATG | CCGGATCAGGTTGATGTTGT |
| 49 | GR487979 | ATCTCAATCGGCAGACCATC | CGCCTATCTGACGGCTTATT |
| 52 | GR487980 | ACAATCTGAAGCGCTGTTT | GATAGGGCGATCGACTGGTA |
| 53 | GR487981 | AATATTGACTCGGCGACCAT | GCAGGTCTCCGTTTATATCA |
| 54 | GR487982 | TAATGACCTGAATATCCGCTGA | CTTCTGATAGGCCATTGTTTG |
| 58 | GR487983 | GCGCGTATTTTATTTTCCA | CGCATCATGCTACCATTCAC |
| 63 | GR487984 | TGATTGTTACCGCTGCCATA | AGGATAGCGACCTGGCTTTT |
| 64 | GR487985 | GCCTTCAGCACATCACCTTT | TTGACATTAGCGGAGCACTG |
| 65 | GR487986 | CAGGAGAAAGCGCACTTGG | CCGATCCGCTGATGGATA |
| 66 | GR487987 | CGCAGCATCATTAGAGAGT | CTCGGGTCGTTAGCTCAGTT |
| 68 | GR487988 | GTCAGCACAGGCGGTAACCTG | ACGGCTCTGCAGGTGATG |
| 69 | GR487989 | CGCGGTCAAGTTTACCTCTC | TGGCAATCGATCTGATGAAA |
| 71 | GR487990 | CGGTGAACGCGCAGTATATC | GCAGCATCGCCTCACACT |
| 80 | GR487991 | GGTTACCGTCTGAACGCTGT | GTTAGCGGTGGCATTACGTT |
| 81 | GR487992 | GGACTGCCGAATGAAACAAT | GTGCCAAAATAGCCACCAT |
| 85 | GR487993 | GTGAGTGAGGTTGCCGAGAT | TGCCCGGATTAAACCAATTTA |

differentiation of hemopoietic cells [36]. Immunization with recombinant G6PI has been demonstrated to be able to induce T-cell-dependent peripheral polyarthritis in mice [37]. Our results of G6PI's consistent overexpression (4–8-fold) in the modified live vaccine strain suggests that G6PI might be one of the factors involved in the protective immunity induced by the vaccine strain.

EST GR487960 shared 88% homology with D-alanyl-D-alanine peptidase of *Yersinia pestis* biovar *Orientalis* (e value = $1e-16$). D-Alanyl-D-alanine peptidase, encoded by gene *VanX*, is critical for vancomycin-resistant enterococci (VRE) [38]. The action of the peptidase will result in hydrolysis of the cellular pool of D-alanyl-D-alanine peptides [39]. Thus, with the severe reduction in cellular D-alanyl-D-alanine peptides, the growing cell wall peptidoglycan termini would instead be incorporated with D-alanyl-D-lac, resulting in dramatic reduction in the affinity of vancomycin to the altered cell wall termini (D-alanyl-D-lac instead of D-alanyl-D-alanine) (up to 1000-fold), accounting for the observed phenotypic resistance [39]. As a result, the peptide-strand cross-linking effect of vancomycin leading to lysis and cell death is prevented in VRE. Our results of consistent upregulation

(5–7-fold) of D-alanyl-D-alanine peptidase in the modified live vaccine strain suggest that D-alanyl-D-alanine peptidase might play a critical role in the resistance to rifampicin, the antibiotic used for the selection of this vaccine strain.

EST GR487978 shared 91% homology with outer membrane protein assembly factor YaeT of *E. fergusonii* (e value = $2e-12$). It has been reported that deletion mutant of *ompA*, a small outer membrane lipoprotein that is a component of the essential YaeT outer membrane, is more sensitive than its wild-type parent *S. enterica* serovar *typhimurium* (*S. typhimurium*) to growth in the presence of hydrophobic antibiotic rifampicin [40]. Our results revealed that YaeT was consistently upregulated (3–6-fold) in the modified live vaccine strain of *E. ictaluri*, suggesting that YaeT might play an important role in the vaccine's resistance to rifampicin.

EST GR487975 shared 98% homology with phosphatidylserine synthase (PSS) of *E. ictaluri* (e value = $3e-35$). The function of PSS is to catalyze a transphosphatidyltransfer reaction to produce phosphatidylserine (PS), a phospholipid. Using fluorescent biosensor, the distribution and

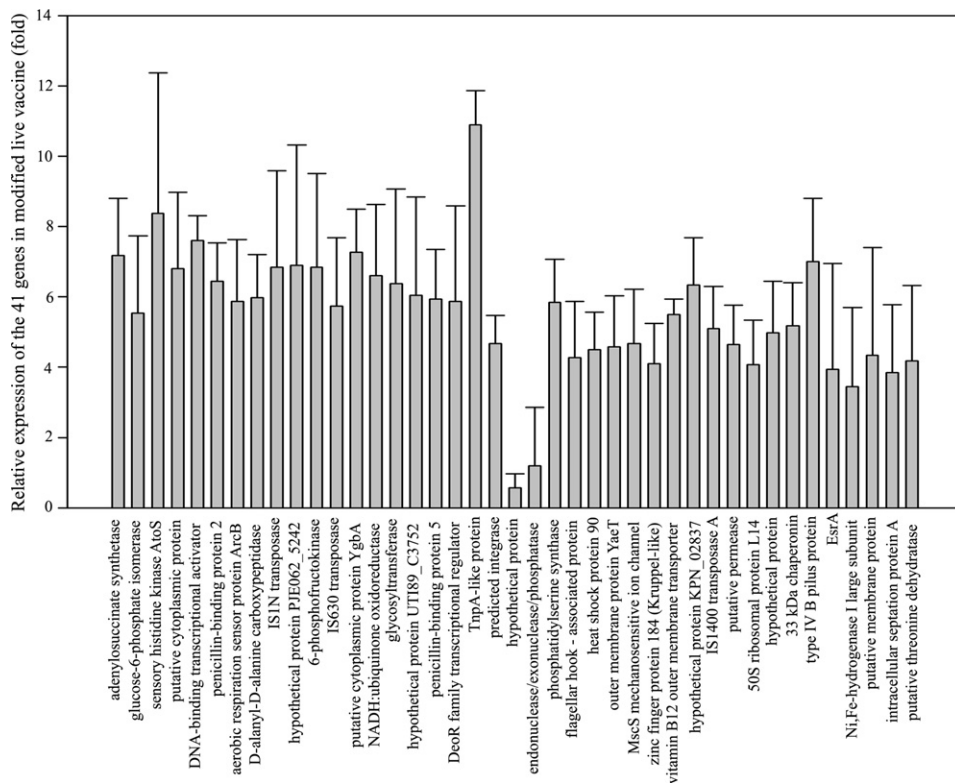


Fig. 1. Transcriptional levels of the 41 genes in the modified live vaccine strain of *E. ictaluri*. The relative expression level of a specific gene in the modified live vaccine strain compared to that in the virulent parent strain was calculated by the formula $2^{\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_t(\text{vaccine}) - \Delta C_t(\text{parent})$ and $\Delta C_t = C_t(\text{gene}) - C_t(16S \text{ rRNA})$. Data are presented as mean \pm S.E.

dynamics of PS during phagosome formation and maturation has been recently monitored [41]. It has been demonstrated that high PS levels are maintained through fusion with endosomes and lysosomes and suffice to attract cationic proteins like *c-Src* to maturing phagosomes [41]. These findings strongly suggest that PS plays a pivotal role in phagosome maturation and microbial killing. Our results revealed that PSS was consistently upregulated (5–7-fold) in the modified live vaccine strain of *E. ictaluri*, suggesting that PSS might also contribute to the protective immunity induced by the vaccine strain.

EST GR487977 shared 78% homology with heat shock protein 90 of *Serratia proteamaculans* (Enterobacteriaceae) (*e* value = $4e-23$). Heat shock proteins (Hsp) mediate a wide range of housekeeping functions in cellular protein biosynthesis such as protein folding, preventing aggregation, or facilitating transmembrane traffic [42]. Hsp molecules activate cells of the innate immune system including antigen-presenting cells (APC) [43]. It has been reported that immunization with either in vivo assembled Hsp/peptide complexes isolated from tumor or virus-infected cells are able to induce potent CD8 T-cell responses that can mediate protective anti-tumor or antiviral immunity. Similarly, vaccines in which antigenic protein domains fused to microbial or murine Hsp molecules are able to stimulate CD8 T-cell immune responses [44–48]. Our results revealed that hsp90 was consistently upregulated (4–6-fold) in the modified live

vaccine strain of *E. ictaluri*, suggesting that hsp90 might be one of the immunogens that contribute to the protective immunity induced by the vaccine strain.

Three virulence-related ESTs (GR487988, GR487989, and GR487967) identified shared 90%, 89%, and 47% homologies with bacterial type IV B pilus, EsrA, and glycosyltransferase, respectively. It has been demonstrated that deletion mutant of type IV pilus protein in *Burkholderia pseudomallei*, the causative agent of melioidosis, has reduced adherence to human epithelial cells [49]. Similarly, inactivation of a putative glycosyltransferase in *Enterococcus faecalis* has led to an almost complete arrest of biofilm formation on plastic surfaces and impaired adherence to cells [50]. On the other hand, overexpression of the putative glycosyltransferase has resulted in increased biofilm production [50]. EsrA, one of *E. tarda* secretion system regulators (*esr*), has been linked to adherence and internalization of *E. tarda* [51]. Our results revealed that these three adhesion and virulence-related genes were upregulated in the modified live vaccine strain, suggesting that increased adhesion might also play important roles in the protective immunity induced by the vaccine strain.

Two ESTs (GR487970 and GR487971) shared homologies with DeoR family transcriptional regulator and TnpA-like protein, respectively. The *E. coli* family of DeoR transcriptional regulators contains at least 14 members. Members of the DeoR family of transcriptional regulators

Table 3Putative function of the 41 genes upregulated genes in the modified live vaccine strain of *E. ictaluri*.

| Protein category | Putative function |
|--|--|
| Immunity-related (9) | |
| Adenylosuccinate synthetase (purA) | Protective immunity |
| Glucose-6-phosphate isomerase | Induces proinflammatory cytokines |
| Penicillin-binding protein 2 | Induces protective immunity |
| D-Alanyl-D-alanine carboxypeptidase | Antibiotic resistance, survival, and immunity |
| <i>E. coli</i> dacA gene, penicillin-binding protein 5 | Protective immunity |
| Phosphatidylserine synthase | Evasion mechanism and immunity |
| Flagellar hook-associated protein | Protective immunity |
| Heat shock protein 90 | Protective immune response |
| 33 kDa chaperonin | Protective immunity |
| Adhesion and virulence-related (3) | |
| Type IV B pilus protein | Adhesion and virulence |
| EsrA | Adhesion and invasion rate |
| Glycosyltransferase | Adhesion and virulence |
| Growth and survival-related (8) | |
| 6-Phosphofructokinase | Survival and acid resistance |
| Outer membrane protein assembly factor YaeT | Outer membrane assembly and growth in rifampicin |
| Aerobic respiration control sensor protein ArcB | Anaerobic growth |
| Vitamin B12 outer membrane transporter | Growth and survival |
| MscS mechanosensitive ion channel | Stress relief |
| Putative permease | Overexpressed in salt-stressed cells |
| 50S ribosomal protein L14 | Expressed under heat stress after LPS injection |
| NADH:ubiquinone oxidoreductase subunit C/D | Provides motility |
| Signal transduction (3) | |
| Sensory histidine kinase AtoS | Signal transduction |
| DNA-binding transcriptional activator CadC | Acid and lysine signaling |
| Putative NBS-LRR protein | Disease resistance signaling |
| Metabolism (2) | |
| COG0374: Ni,Fe-hydrogenase I large subunit | Hydrogen sensor and hydrogen metabolism |
| Putative threonine dehydratase | Serine metabolism |
| Transcriptional regulation (2) | |
| DeoR family transcriptional regulator | Transcriptional regulation |
| TnpA-like protein | Transcriptional regulation |
| Unknown function (8) | |
| Putative cytoplasmic protein | Unknown |
| Hypothetical protein PJE062_5242 | Unknown |
| Putative cytoplasmic protein YgbA | Unknown |
| Hypothetical protein UTI89_C3752 | Unknown |
| Hypothetical protein KPN_02837 | Unknown |
| Hypothetical protein | Unknown |
| Putative membrane protein | Unknown |
| Intracellular septation protein A | Unknown |
| Ungrouped protein (6) | |
| Predicted integrase | Ungrouped |
| Endonuclease/exonuclease/phosphatase | Ungrouped |
| IS1N transposase | Ungrouped |
| IS630 transposase | Ungrouped |
| Zinc finger protein 184 (Krüppel-like) | Ungrouped |
| IS1400 transposase A | Ungrouped |

are present in a variety of bacterial organisms, ranging from Gram-positive bacteria to Gram-negative bacteria. It has been demonstrated that DeoT, a DeoR-type transcriptional regulator, is able to regulate the expression of a number of genes involved in a variety of metabolic pathways including transport of maltose, fatty acid oxidation and peptide degradation [52]. TnpA is a weak transcriptional activator of suppressor–mutator (Spm), an autonomous transposable element from maize that encodes several alternatively spliced transcripts [53]. Two Spm-encoded proteins, TnpA and TnpD, are essential

for excision and transposition of the element [53]. The overexpression of TnpA-like protein and DeoR family transcriptional regulator suggests that they might regulate gene expression in the vaccine strain.

Although majority of the genes identified from the subtractive library shared certain homologies with proteins deposited in the gene bank, a large portion (22%) of them was either putative or hypothetical. Our results revealed that some of them were consistently upregulated in the modified live vaccine strain. For example, the transcriptional level of EST GR487956 was

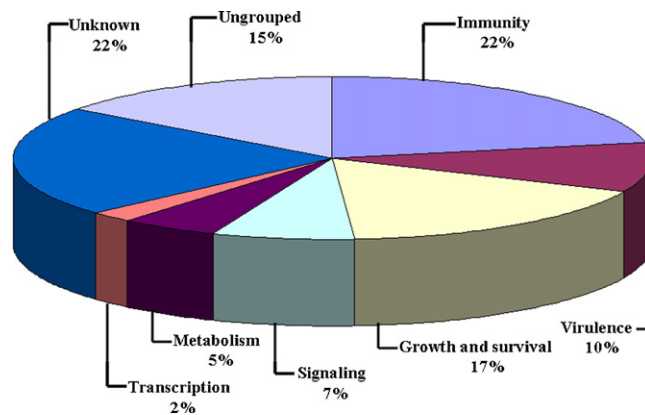


Fig. 2. Classification of the differentially expressed genes identified from the subtractive library. Pie charts representing the distribution of the 41 identified genes according to their putative biological function.

5–9-fold higher in the modified live vaccine strain than that in the virulent parent strain. EST GR487956 shared only 65% identity with a putative cytoplasmic protein of *S. typhimurium* with a very high *e* value (33.5). However, EST GR487956 shared 91% identity with *E. ictaluri* isolate 93-146 O antigen biosynthesis gene cluster at nucleotide level with a very low *e* value (6e–18), suggesting that EST GR487956 might be related to O antigen biosynthesis. The upregulation of such genes in the vaccine strain merits functional studies for them in inducing protective immunity in fish.

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